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REVIEW

# Reactive oxygen species and the neuronal fate

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**Abstract** Aberrant or elevated levels of reactive oxygen species (ROS) can mediate deleterious cellular effects, including neuronal toxicity and degeneration observed in the etiology of a number of pathological conditions, including Alzheimer's and Parkinson's diseases. Nevertheless, ROS can be generated in a controlled manner and can regulate redox sensitive transcription factors such as NF$\kappa$B, AP-1 and NFAT. Moreover, ROS can modulate the redox state of tyrosine phosphorylated proteins, thereby having an impact on many transcriptional networks and signaling cascades important for neurogenesis. A large body of literature links the controlled generation of ROS at low-to-moderate levels with the stimulation of differentiation in certain developmental programs such as neurogenesis. In this regard, ROS are involved in governing the acquisition of the neural fate—from neural induction to the elaboration of axons. Here, we summarize and discuss the growing body of literature that describe a role for ROS signaling in neuronal development.

**Keywords** Neurogenesis · Stem cell · Reactive oxygen species · Gene expression · Signaling

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## Introduction

Reactive oxygen species (ROS) are highly reactive metabolites of oxygen ($O_2$) that include hydrogen peroxide ($H_2O_2$), superoxide anion ($O_2^{\cdot-}$) and the hydroxyl radical ($^{\cdot}OH$). Typically, ROS are associated with cell malfunction and disease. Indeed, high levels of ROS have been linked to several neurological disorders, including Parkinson's and Alzheimer's diseases, cell death and senescence [1, 2]. However, there is a growing body of literature supporting crucial roles for ROS in the regulation of cell signaling [3–5], growth [6–11], $Ca^{2+}$ signaling [12–15], adhesion, and control of redox sensitive gene expression [3], in addition to the well-documented role of host defense. The concept that redox status might also play an important role in neurogenesis has become a growing area of research in recent years. This review will first provide a brief overview of the process of neurogenesis in the embryo as well as recent work in the ROS field, focusing on studies supporting a role for ROS in neuronal development.

## Overview of neurogenesis

In the vertebrate embryo, neuronal induction occurs in a subset of cells in the ectoderm in response to the inhibition of transforming growth factor beta (TGF$\beta$) and Wnt, as well as the activation of fibroblast growth factor (FGF) signaling. The tissue derived is called the neural plate (reviewed in [16]). The neural plate is transformed into the neural tube. Within the neural tube, neurogenesis takes place in the cells of the germinal neuroepithelium (NE) or ventricular zone (VZ). These cells undergo proliferative and differentiating cell divisions to generate neural precursors (neuroblasts), neurons and glial cells that populate

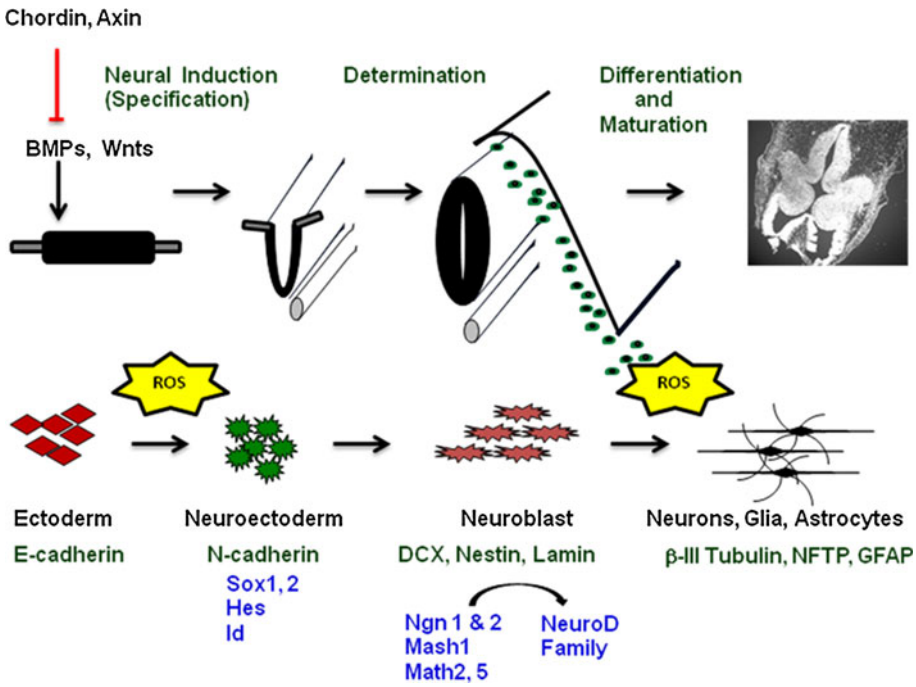


**Fig. 1** A schematic showing the steps involved in the morphogenesis and transcriptional control of neurogenesis. Inductive signals that include inhibitors of BMP and Wnt mediate the transition of epithelial cells into neuroepithelial cells. This is marked by the sequential upregulation of factors such as Sox 1–3 and Hes1 and 5 which mark the neuronal precursor population. The bHLH transcription factors Ascl1 (Mash1), Neurogenin1–3 and Math and NeuroD are upregulated next. They mark committed neuroblasts. Their expression is a hallmark of the activation of neurogenesis. The neural plate is transformed into the neural tube. Subsequently, pseudostratified epithelial cells within the neural tube multiply and differentiate into distinct neural lineages. Neuronal structural proteins such as $\beta$III-Tubulin and Neurofilament are expressed by postmitotic neurons. Reactive oxygen species are involved in the molecular mechanisms that govern the acquisition of the neural fate, from early steps to the elaboration of axons [17–21]

the central nervous system. Postmitotic neurons and neuroblasts migrate to the marginal zone and beyond to assume their terminal fate [17–21].

Glial cells, astrocytes and oligodendrocytes are crucial for growth and homeostasis of neurons. They are produced in the nervous system along with neurons in overlapping but defined temporal windows [22–26]. Together, these cells differentiate to generate several hundred different neurons and support cells that make up the central nervous system (CNS), and the peripheral nervous system (PNS) of the adult brain [27].

Transcription factors related to the *Drosophila* genes *achaete-scute* (*as-c*) [28, 29] and *atonal* (*ato*) family [29] conservatively regulate neuronal development in vertebrates. The upregulation of these basic helix-loop-helix (bHLH) family of transcription factors is a hallmark of the activation of neurogenesis. Mash1, Neurogenin, Math and NeuroD are termed proneural factors [28, 30–32]. These factors heterodimerize with ubiquitously expressed bHLH factors, called E proteins in vertebrates and Daughterless (Da) in *Drosophila*), to induce neurogenesis [33–36]. Mash1, Neurogenin1 and Neurogenin2 are early-acting. They activate NeuroD family members to induce pan and sublineage-specific neuronal differentiation. Math1 and 5 function in neuronal determination and differentiation, respectively (reviewed in [37, 38]).

Neurogenesis is negatively regulated by the sex determining region of the Y (Sry) chromosome, related to high mobility group (HMG)-containing box (Sox) factors, hairy enhancer of split (Hes) bHLH proteins and Inhibitor of differentiation (Id). Hes and Sox actively repress neuronal differentiation and are expressed in neuronal precursor cells. This inhibition allows for adequate proliferation of precursor cells prior to differentiation. Inhibitory proteins are also utilized to switch from neuron to glial production, and are therefore critical for the diversification and correct patterning of the neuroectoderm (reviewed in [39]). Transcription factors responsible for the expression of proneural and neuronal inhibitory genes are downregulated upon terminal neuronal differentiation [40], which is marked by the appearance of generic and/or sub-type specific neuronal structural proteins such as Doublecortin, $\beta$III-Tubulin and Neurofilament [41, 42]. The major molecular players involved in the orchestration of the neuronal fate are summarized in Fig. 1.

Neuronal cell types can be generated in vitro from pluripotent embryonal carcinoma (EC) and embryonic stem (ES) cells, which are derived from the inner cell mass

(ICM; reviewed in [16]). Additionally, several types of neural stem (NS) cells can be obtained from multipotent embryonic cells or the postnatal brain (reviewed in [43–45]). Since the temporal pattern of in vitro neuronal differentiation in ES cells closely recapitulates in vivo neurogenesis, an abundance of knowledge has been gleaned using these kinds of models.

However, despite the undisputed importance of the above-mentioned transcription factors and signals known to regulate neurogenesis, ROS have been proposed to also play important roles in differentiation. While high levels of ROS have been clearly linked to several neurological disorders, the emerging scenario is that, at controlled levels, ROS can play a positive role in differentiation and development (Fig. 1). The remainder of this review will focus on the emerging importance of ROS in neurogenesis.

## Cellular sources of ROS in the developing nervous system

The role of mitochondrial electron transport chain enzymes in ROS production is well documented. However, data are starting to emerge implicating NADPH oxidase (Nox) family members as additional sources of ROS in many cell types. The Nox enzymes, which were originally discovered in phagocytic cells, are responsible for the so-called 'respiratory burst' upon immune system stimulation by pathogens. In phagocytic cells, the Nox enzyme complex consists of two membrane components, the glycosylated catalytic subunit $gp91^{phox}$ (Nox2) and its dimerization partner $p22^{phox}$. Four cytosolic regulatory subunits ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and the small GTP-binding proteins Rac1 and Rac2) make up the rest of the active complex. Interestingly, several other Nox members have been characterized in non-phagocytic cells [including Nox1, Nox3, Nox4, Nox5 and Dual oxidases 1 and 2 (Duox1 and Duox2)], reviewed in [46]). Microglia, (the immune cells of the brain), are a major source of NADPH oxidase in the nervous system. These NADPH oxidases have been implicated as essential components of normal brain homeostasis, and the perturbation of their signaling leads to deleterious effects in the nervous system [47, 48].

Nox family members Nox1, Nox2, Nox3 and Nox4 [49, 50] have been identified throughout differentiation of pluripotent mouse ES cells, albeit at varying levels. Although full characterization of these enzymes in neurogenesis is lacking, data suggest that they display distinct temporal expression patterns. Nox4 may be of particular interest since it appears to be expressed at robust levels in both dividing mouse ES cells and differentiating embryoid bodies. Although the early expression of Nox4 suggests that it may play an important role in differentiation, more studies will need to be completed to determine the importance of these Nox isoforms in neurogenesis.

The Dual oxidase subgroup of Nox enzymes, which are the most recently discovered family members [51], are unique in that they require maturation factors (DuoxA/Nip) for ROS production in cells. Unlike Nox1–Nox4, dual oxidases do not associate with $p22^{phox}$ and nor do they require the other regulatory units typical of other family members. Duox1 and Duox2 (and their respective maturation factors) were found to be expressed in a variety of adult tissues including the thyroid, airway tracts, salivary glands and the gastro-intestinal tract [52]. Recently, our laboratory detected these enzymes in the developing embryonic and postnatal mouse brain [53]. The structure of Doux features a canonical C-terminal Nox catalytic domain, a bis-heme peroxidase-like domain, and a $Ca^{2+}$-binding domain at the N-terminal region [54, 55]. Human Duox enzymes, like Nox5, contain a calcium binding domain with two EF hands (instead of the canonical four EF hands characteristic of Nox5). The $H_2O_2$ produced by the Duox enzymes is utilized in thyroid hormone biosynthesis. In this process, $H_2O_2$, which acts as the final electron acceptor, facilitates the thyroperoxidase-catalyzed iodination of thyroglobulin and the coupling of iodinated tyrosyl residues via phenoxy-ether bond formation [56, 57]. Agonists capable of increasing intracellular $Ca^{2+}$ levels have been shown to cause corresponding elevations in ROS in thyroid cells [58, 59]. The Duox enzymes can also generate superoxide presumably via their peroxidase domain. Roles for this unique mode of $H_2O_2$ generation and utilization by Duox enzymes have not been fully explored.

The other common source of ROS production in cells is the mitochondrial electron transport chain. In the mitochondria, $O_2$ is converted to $O_2^{\cdot-}$ by complex I (NADH/Ubiquinone oxyreductase) and complex III (ubiquinol/cytochrome *c* oxidoreductase) components. This $O_2^{\cdot-}$ can be converted to $H_2O_2$ by the enzyme superoxide dismutase (SOD). Other minor sources of ROS-producing enzymes in cells include peroxidases and oxygenases. ROS generation is triggered in many cell types in response to stimuli such as cytokines, hormones, growth factors, integrin signaling, hypoxia and differentiation. For example, elevations in ROS levels are associated with cytokines, interleukin1, and tumor necrosis factor in fibroblasts [60, 61]. Furthermore, the correct balance of ROS production and utilization is critical to the regulation of self-renewal and differentiation in pluripotent cells [62–64].

## Regulation of neurogenesis by ROS

ROS have been long known to induce apoptosis and negatively impact neuronal cell fate [65–67], but a growing

body of literature has started to implicate ROS in neurogenesis as opposed to neurodegeneration. Early evidence of beneficial roles for ROS in neurogenesis include the finding that neuroblastoma cells differentiate into neurons in high concentrations of oxygen in vitro [68], and that oxygen pressure and consumption in the nervous system increase during the first few postnatal days, coinciding with terminal differentiation of neurons [69, 70]. Additionally, PC12 cells grown in 50% $O_2$ (hyperoxia) show levels of neuronal differentiation similar to those demonstrated with nerve growth factor (NGF) treatment (e.g., neurite extension, expression of differentiation markers, and tyrosine hydrolase). This phenotype was also observed when these cells were treated with xanthine/xanthine oxidase (which generates $H_2O_2$ and $O_2^{\cdot-}$). Furthermore, treatment with two different antioxidants (ascorbic acid and *N* acetyl L-cysteine), abolished the hyperoxia-induced neurite extension and differentiation [71]. Suzukawa et al. [72] also showed that the increase in ROS, neurite outgrowth, tyrosine phosphorylation and AP1-activation during NGF stimulation of PC12 cells was abrogated by ROS inhibitors and by overexpression of a dominant negative Rac [72].

Neuregulin (NRG), which binds and activates receptor tyrosine kinases ErbB-3 and ErbB-4, increased ROS and induced neuronal differentiation when overexpressed in PC12 cells. Inhibition of ROS (by *N* acetyl L-cysteine) reduced the NRG-induced activation of RAS, Erk and PC12-ErbB-4 cell differentiation [73]. Embryonic rat cortical cells stimulated with FGF2 (which induces a neuronal morphology) display higher ROS levels than in progenitor cells. Cell-permeant versions of both SOD and catalase were able to reduce ROS levels, thus implicating both $H_2O_2$ and $O_2^{\cdot-}$ [74].

Recently, our laboratory showed that ectopic *nip1* expression in mouse embryonal carcinoma P19 cells led to elevated ROS and the upregulation of several neuronal differentiation markers and proneural genes. Nip and Duox are expressed in the neuronal lineage in stem cells as well as the developing brain. Depletion of *nip1* expression by short hairpin RNA led to reductions in neuronal differentiation and ROS generation [53]. Since $H_2O_2$ is known to stimulate neuronal differentiation [71–75], our work provided the first demonstration that Nip can regulate neurogenesis in stem cells by regulating the cellular levels of $H_2O_2$. Hence, we proposed a new mechanism that guides neuronal differentiation and characterized Nip1-mediated ROS production as a novel intrinsic regulator of neuronal cell fate in stem cells [53].

Four alternatively spliced variants of *Nip1* have been described to date. Duoxa1$\alpha$/DuoxA1-2, Duoxa1$\beta$/DuoxaA1-1, Duoxa1$\gamma$/DuoxA1-3, and Duox1$\delta$ contain 343, 298, 483 and 438 amino acids, respectively. These isoforms differ in their ability to target Duox to the plasma membrane or to internal membranes, and in the nature of ROS released. Interestingly, only the DuoxA1$\gamma$/DuoxA1-3 isoform ($\sim$55 kDa) which shows closest homology to *Drosophila* Nip [76], and the DuoxA1$\alpha$ variant ($\sim$40 kDa) which is closest in sequence to DuoxA2, were found to support hydrogen peroxide generation and optimal maturation of Duox1 and Duox2 [77, 78]. The role of Nip in homeostasis may be to integrate functions in the developing brain and endocrine systems. This is plausible since several studies report that alterations in thyroid hormone signaling components can modulate neuronal development (reviewed in [79]). Therefore, Nip, a component of the ROS-generating Duox system and an interaction partner of the cell fate determinant Numb, likely has a profound influence in the development of pluripotent cells into the neuronal lineage. Additionally, work supporting roles for other NADPH oxidase family members in neuronal differentiation is starting to emerge. It was recently demonstrated that PKC is stimulated by NADPH oxidases during the retinoic acid-induced neuronal differentiation of neuroblastoma cells [80].

Along with growing evidence in support of a role for ROS in neurogenesis, recent work also suggests that endogenous ROS levels fluctuate throughout differentiation [81]. It is also becoming apparent that cells produce different levels of ROS depending on their developmental stage, and thus have varying degrees of tolerance for oxidative stress [81]. It has been recently determined that exposure to exogenous ROS enhances self-renewal and differentiation of proliferative neural progenitors (which exhibit higher endogenous levels of ROS than their differentiating counterparts), but results in toxicity in differentiating neurons [81]. Additionally, in another recent study, quantification of intracellular redox status using mass spectrometry revealed that dynamic changes in the redox balance occur during the transition from pluripotent cells to differentiated neurons. Differentiated neurons were found to be in enriched in saturated fatty acids, including eicosanoids, neuroprotin D1, leukotrien B4 and metabolites that undergo mitochondria beta oxidation. Gain-of-function studies demonstrated that these candidate factors could increase the presence of neuronal markers in stem cells [82].

While the exact mechanisms involved in ROS-mediated neurogenesis remain unclear, it has been previously established that endogenously produced $H_2O_2$ activates PI3K/Akt [81], p38 MAPK [49] and ERK [83] signaling. Similarly, ROS are known to stimulate nuclear factor kappa light chain enhancer of activated B cells (NF$\kappa$B) [84]. The recent discovery that the activation of two other ROS-dependent transcription factors [activator protein 1 (AP-1) and nuclear factor of activated T cells (NF-AT)] is decreased upon knockdown of Duox1 in Jurkat T cells [3],

supports the possibility that ROS-mediated differentiation results from the activation of well-known signaling pathways and redox-sensitive transcription factors. Future research will continue to reveal not only the signaling cascades initiated by ROS but also the importance of their activation during neurogenesis.

## Conclusion and future directions

Recent findings provide convincing evidence for a role of ROS in the regulation of the neuronal fate. What remains puzzling are the sheer number of enzymes (different Nox and Duox isoforms) expressed in neural progenitor cells, all of which are capable of producing ROS. Studies in mouse tissues and mouse embryonic stem cells also reveal an abundance of these enzymes in these systems [49, 50, 53]. Attempts at characterizing Nox and Duox proteins suggest differences in temporal expression during differentiation, as well as differences in the resulting phenotypes from their knockdown or overexpression. A new level of complexity is added when one considers that these enzymes may be activated by different stimuli, they may signal through different pathways, and are likely not fully redundant. Indeed, much about our understanding regarding the effects of ROS on differentiation remains unknown. Whether the careful manipulation of ROS-producing enzymes may be useful in the future treatment of neuro-degenerative diseases is also unclear. However, links between ROS and differentiation represent important discoveries in light of the fact that high levels are known to be catastrophic to cells, and ROS generating systems are now being considered as potential targets in the development of stem cell therapies [83].

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